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Structural analysis of recombinant von Willebrand factor produced at industrial scale fermentation of transformed CHO cells co-expressing recombinant furin

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Abstract Thorough analysis of multimer composition and molecular structure of recombinant von Willebrand factor (r-vWF) produced by recombinant CHO cells demonstrated r-vWF to be more intact and less proteolytically degraded than plasma-derived vWF (pd-vWF) [B. Fischer et al. (1994) FEBS Lett. 351, 345–348]. In contrast to pd-vWF, r-vWF preparations consisted of pro-vWF (vWF containing covalently attached propeptide) as well as mature vWF subunits forming homo- and hetero-multimers. In order to ensure complete propeptide processing, a r-vWF-producing CHO cell clone was transfected with the cDNA of the human propeptide processing enzyme Furin. A r-vWF/r-Furin co-expressing cell clone was cultivated at industrial scale in high cell density perfusion fermenters. r-vWF obtained from these cells was fully processed. Analysis of r-vWF by multimer analysis revealed a multimer pattern equal in number of high molecular weight multimer to pd-vWF, but absence of satellite bands. Two-dimensional electrophoretic analysis of both the primary dimer and the complete multimer pattern of r-vWF showed that the recombinant coagulation factor was composed exclusively of intact and mature subunits. Since the triplet structure typical to pd-vWF is known to reflect proteolytic degradation, r-vWF thus exhibits an integrity far superior compared to pd-vWF.

Key words: Von Willebrand factor; Furin; Recombinant expression; Multimer structure

1. Introduction

The adhesive protein von Willebrand factor exists in human plasma as a series of heterogeneous homo-multimers ranging in size from about 450 kDa to more than 10,000 kDa [1–4]. The precursor polypeptide produced in endothelial cells, pre-pro-vWF, consists of a 22-residue signal peptide, a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma vWF. After removal of the signal peptide, the resulting pro-vWF subunits are engaged in a complex biosynthetic process thought to begin with the formation of a primary dimer, containing two pro-vWF subunits, through disulphide bond linkage. Then protomeric units of the multimeric series are assembled into higher order multimers by disulphide bonding of dimers. vWF pro-peptide is cleaved from multimeric vWF before release from intracellular storage sites into circulation [1,3,5,6]. In human plasma vWF serves as a carrier for coagulation factor VIII and is required for adhesion of platelets to the endothelium of injured vessel walls.

Plasma concentrates containing vWF are used in the therapy of patients with von Willebrand disease unresponsive to the nontransfusional agent desmopressin. However, therapeutic plasma concentrates containing vWF (i) lack the largest, most hemostatically active multimers, and (ii) exhibit various degree of degradation of vWF [7]. Apparently, the structural damage to plasma-derived vWF is caused by soluble and platelets/leucocytes associated proteases and occurs at early stages of the manufacturing process of plasma concentrates [7]. Changing qualities of donor blood and different preparation strategies make it even more complicate to produce plasma-derived vWF of equal high quality from batch to bach. However, these problems may be solved by production of recombinant vWF (r-vWF) by fermentation of recombinant cells on an industrial scale.

In a previous investigation, wild-type vWF full-length cDNA was expressed in CHO cells (cell clone # 808). Recombinant vWF (r-vWF/808) was obtained and its molecular structure was investigated to characterize the polypeptide composition of r-vWF [8]. Results demonstrated r-vWF/808 to be less proteolytically degraded than pd-vWF, but to contain significant amounts of pro-vWF subunits. Analysis of the primary dimer of r-vWF/808 identified homo-dimers of either two pro-vWF subunits or two mature vWF subunits, as well as hetero-dimers of pro-vWF and mature vWF subunits.

Here, r-vWF-producing CHO cells were additionally manipulated to co-express the recombinant subtilisinlike proprotein processing enzyme Furin (r-Furin) [9,10] in order to ensure complete processing of pro-vWF. Recombinant CHO cells (cell clone # 904) were cultivated for the first time on an industrial scale in high cell density perfusion bio-reactors. Multimer composition and molecular structure of r-vWF/904 produced from r-vWF/r-Furin cells were investigated.

2. Materials and methods

2.1. Materials

Seakem Agarose HGT(P) and Gel Bond Film were purchased from FMC BioProducts. Acrylamide, *N,N'*-methylene-bis-acrylamide, sodium dodecyl sulfate (SDS), glycine, ammonium persulfate and alkaline phosphatase coloration chemicals were from Bio-Rad Laboratories. Normal plasma was from Immuno AG. Rabbit anti-human vWF polyclonal antibody and alkaline phosphatase conjugated anti-rabbit polyclonal antibody were from Dakopatts. Alkaline phosphatase conjugated affinity purified goat anti rabbit IgG (H + L) was obtained from Axell Accurate Chemical. Enzyme-linked immunoadsorbent assay for vWF was from Boehringer Mannheim. Cytoline 2 microcarriers were from Pharmacia. All other reagents were purchased from Sigma.

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2.2. Expression of recombinant von Willebrand factor

Establishment of r-vWF-producing CHO cell clone # 808 was described previously [8]. By transfection with an expression vector encoding the human Furin cDNA [11] these cells were additionally manipulated to over express recombinant Furin, yielding cell clone # 904 producing r-vWF/904. For scale up production, doubly transformed CHO cells were cultivated on an industrial scale in high cell density perfusion bio-reactors [12]. Cytoline microcarriers were used for the culture of transformed CHO cells in 40 liter bio-reactors.

2.3. SDS-agarose gel electrophoresis

SDS-agarose gel electrophoresis was performed in a horizontal electrophoresis system modified according to Ruggeri and Zimmermann [2] using low resolution 1% agarose gels and high resolution 2% agarose gels. Visualization of vWF multimers was carried out by immunoenzymatic staining modified according to Aihara et al. [13]. As primary antibody a rabbit anti human von Willebrand factor anti serum was used in a dilution of 1:5000. As a secondary antibody alkaline phosphatase conjugated affinity purified goat anti rabbit IgG was used in a dilution of 1:1000. Staining was performed with the nitroblue tetrazolium chloride/bromochloro indolyl phosphate substrate system.

2.4. Two-dimensional SDS-electrophoresis

High resolution two-dimensional SDS-electrophoresis of vWF primary dimer was performed by combination of non-reducing high-resolution SDS-polyacrylamide/agarose gel for the first dimension and reducing SDS-polyacrylamide gel for the second dimension. Preparation of first dimension non-reducing SDS-3% polyacrylamide/0.5% agarose gels with 5% crosslinking and sample preparation have been described in detail previously [8]. For resolution in the second dimension, 5% acrylamide SDS-polyacrylamide gels under reducing conditions were prepared [8]. Two-dimensional SDS-electrophoresis of vWF multimers was performed by combination of non-reducing low-resolution SDS-1% agarose gel for the first dimension and reducing SDS-5% polyacrylamide gel for the second dimension.

2.5. Immunoblotting

Polypeptides resolved by electrophoresis were transferred onto nitrocellulose membranes in 25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol buffer [14]. Detection of vWF by rabbit anti-human vWF immunoglobulins and staining by alkaline phosphatase conjugated goat anti-rabbit antibodies has been described previously [8].

3. Results and discussion

Preparation of plasma-derived vWF for therapy of patients with von Willebrand disease is limited due to lack the largest, most hemostatically active multimers, and proteolytic degradation of vWF during plasma fractionation [7]. An alternative to plasma-derived protein may be the production of recombinant vWF.

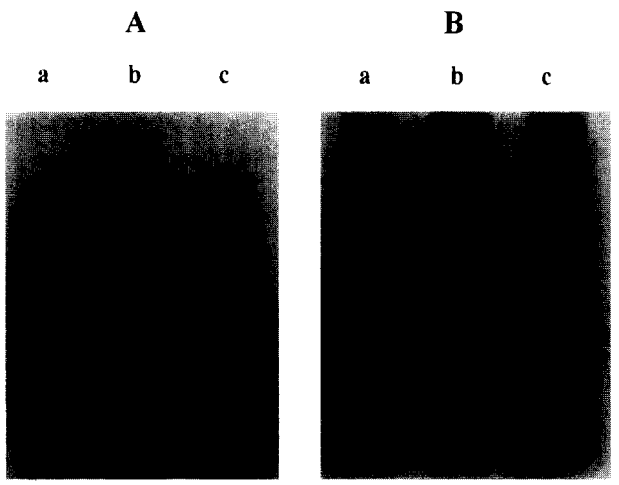

Fig. 1. SDS-agarose gel electrophoresis of von Willebrand factor at non-reducing conditions. (A) Low resolution 1% agarose gel electrophoresis. (B) High resolution 2% agarose gel electrophoresis. (a) pd-vWF, (b) r-vWF/904, (c) r-vWF/808.

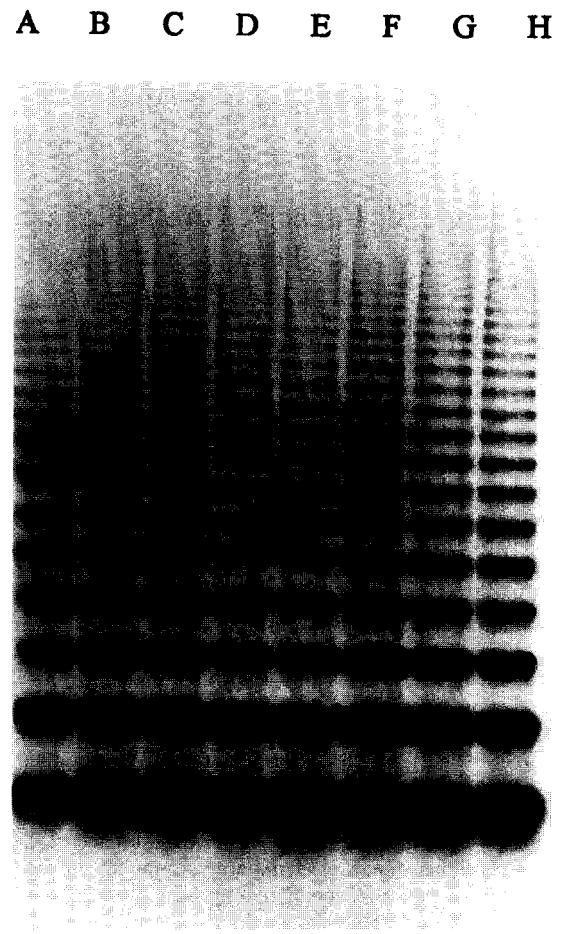

Fig. 2. Multimer analysis of r-vWF/904 during adaptation of recombinant CHO cells to serum-free medium. (A) r-vWF/904 produced at day 18 in fermentation medium containing 2.5% serum; (B) r-vWF/904 produced at day 24 during adaptation to serum free medium; (C) r-vWF/904 produced at day 29 during adaptation to serum free medium; (D) r-vWF/904 produced at day 35 in serum-free medium; (E) r-vWF/904 produced at day 44 in serum-free medium; (F) r-vWF/904 produced at day 51 in serum-free medium; (G) r-vWF/904 produced at day 56 in serum free medium; (H) r-vWF/904 produced at day 65 in serum-free medium.

In a previous investigation structural properties of r-vWF/808 produced by recombinant CHO cells have been described [8]. Due to incomplete processing, r-vWF/808 contained significant amounts of pro-vWF thus forming homo- and heteropolymers. Here we present structural data on r-vWF/904 produced on an industrial scale by recombinant CHO cells secreting both recombinant vWF and recombinant propeptidase Furin. To ensure complete processing, r-vWF-producing CHO-cells (cell clone # 808; producing r-vWF/808) were additionally manipulated to co-express the recombinant propeptidase Furin, resulting in CHO-cell clone # 904, producing r-vWF/904. Fig. 1 compares the multimer structures of r-vWF/808, r-vWF/904 and pd-vWF, at low resolution 1% agarose gel electrophoresis (Fig. 1A) and high resolution 2% agarose gel electrophoresis (Fig. 1B). Both pd-vWF, r-vWF/808 and r-vWF/904 exhibited a similar multimer pattern. However, individual

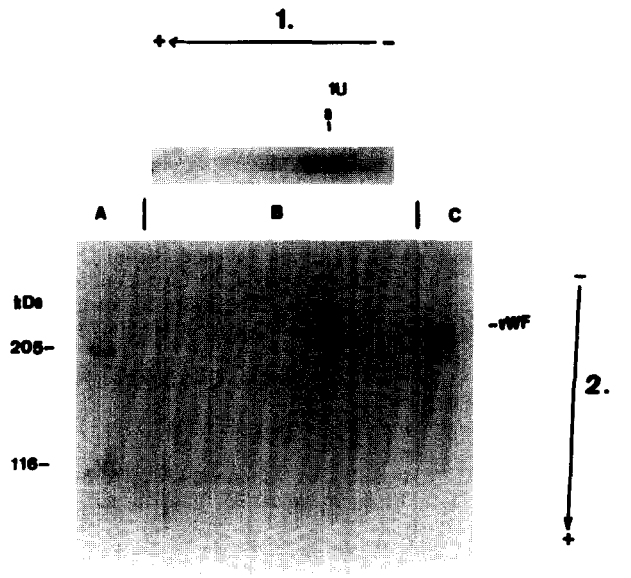

Fig. 3. Two-dimensional analysis of the primary dimer of r-vWF/904. r-vWF/904 was separated in the first dimension by non-reducing high resolution SDS-3% acrylamide/0.5% agarose gel electrophoresis. Individual gel lanes containing the primary dimer vWF-related proteins were cut out, incubated in reducing buffer, mounted on top of a 5% acrylamide gel, and electrophoresed in the second dimension (B). Simultaneously, denatured-reduced r-vWF/904 (C), and prestained reference proteins (A) were analysed.

multimers of r-vWF/904 appear to be more compact than to pd-vWF and r-vWF/808 multimers. Individual pd-vWF multimers are surrounded by diffuse satellite bands of slightly increased as well as reduced molecular weights, forming a triplet structure typical to pd-vWF. The satellite bands have been identified as proteolytic degradation products of vWF in plasma [15–17]. Protease cleavage sites have been localized in pd-vWF [18]. By contrast, satellite bands are missing both in r vWF/808 and r-vWF/904 (Fig. 1). A somewhat broader picture of individual multimer bands of r-vWF/808 results from the mixtures of various combinations of mature vWF and pro-vWF subunits forming homo- and hetero-multimers of increased moleculare weight [8]. By contrast, electrophoretic analysis under reducing conditions showed that of r-vWF/904 produced on an industrial scale fermentation was composed exclusively of mature vWF monomers, explaining the more compact r-vWF/904 multimer pattern.

Proteolysis of pd-vWF may also result in loss of the largest, most hemostatically active cWF multimers [7,17]. However, both r-vWF/808 and r-vWF/904 exhibited very high molecular weight multimers absent in pd-vWF (Fig. 1).

The possibility of reduced concomitant quantities of pro-vWF during the expression of r-vWF by co-expressing the propeptidase Furin has been tested so far on an laboratory scale. In the present investigation double-transformed CHO cells were cultivated on an industrial scale in high cell density perfusion bio-reactors. During the expression of r-vWF cell density reached levels of $3–5 \times 10^7$ cells per ml carrier. Stable expression of r-vWF/904 over a period of several weeks resulted in the continuous production of several hundred mg vWF antigen per day. Fig. 2 shows the multimer analysis of r-vWF/904 produced by recombinant CHO cells prior, during and after adaptation to serum-free medium. Adaptation to serum-free medium did not affect large-size multimers. More than 20 repeats of multimers are observed for r-vWF/904, ruling out proteolytic digestion of r-vWF/904.

The structural composition of the primary dimer of r-vWF/904 was analyzed by high resolution SDS-3% acrylamide/0.5% agarose gel electrophoresis at non-reducing conditions. The results showed that the primary dimer of r-vWF/904 exhibited a single protein band of a molecular weight of 450 kDa (Fig. 3). In contrast, in previous studies r-vWF/808 was separated into three protein bands with molecular masses between 450 to 600 kDa [8], representing homo-dimers of mature vWF subunits, hetero-dimers of mature vWF and pro-vWF subunits and homo-dimers of pro-vWF subunits [8]. The primary dimer of pd-vWF exhibited a heterogeneous mixture of protein bands with apparent molecular weights of 150 kDa to 450 kDa, reflecting proteolytic degradation of pd-vWF subunits [8]. To analyze the molecular composition of the primary dimer of r-vWF/904 in more detail, two-dimensional electrophoresis was carried out with non-reducing SDS-polyacrylamide/agarose gel electrophoresis in the first dimension and reducing SDS-polyacrylamide gel electrophoresis in the second dimension. High-resolution SDS-polyacrylamide/agarose gel electrophoresis of r-vWF/904 (Fig. 3) resulted in one protein band labeled $1U_a$ (terminology according to [15]) in the first dimension. Second dimension electrophoresis showed that this protein band contained exclusively mature vWF subunits. No proteolytic degradation products were detected. In contrast, previous structural analysis of the primary dimers of r-vWF/808 and pd-vWF the detected hetero- as well as homo-dimers and different vWF-subunit degradation products, respectively [8].

Fig. 4 shows the two-dimensional electrophoretic analysis of the complete multimer pattern of r-vWF/904. First dimension non-reducing SDS-1% agarose gel electrophoresis of r-vWF/904 separated all the individual multimers. Second dimension SDS-5% acrylamide gel electrophoresis under reducing conditions showed that all r-vWF/904 multimers were composed exclusively of intact mature subunits. Although produced in the presence of the protease Furin, r-vWF/904 exhibited no degra-

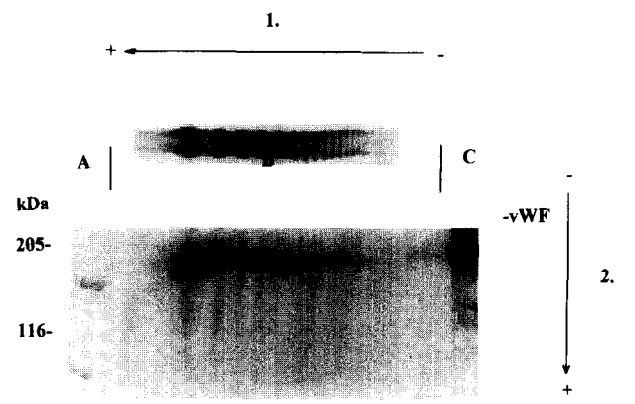

Fig. 4. Two-dimensional analysis of multimer pattern of r-vWF/904. r-vWF/904 was separated in the first dimension by non-reducing SDS-1% agarose gel electrophoresis. An individual gel lane containing the multimer pattern was cut out, incubated in reducing buffer, mounted on top of a 5% acrylamide gel, and electrophoresed in the second dimension (B). Simultaneously, denatured-reduced r-vWF/904 (C), and prestained reference proteins (A) were analysed.

dation products. Thus, stable expression of recombinant vWF in the presence of overexpressed propeptidase Furin, produced on an industrial scale, resulted in a completely processed, maturated vWF, composed of intact subunits forming low and high molecular weight multimers.

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